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Liposome-enhanced transformation of *Streptococcus lactis* and plasmid transfer by intergeneric protoplast fusion of *Streptococcus lactis* and *Bacillus subtilis*

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1. SUMMARY

An efficient protoplast transformation system and a procedure of plasmid transfer by means of protoplast fusion is described for *Streptococcus lactis*. Protoplasts of *S. lactis* IL1403 and *S. lactis* MG1363 were transformed by pGK12 [2.9 MDa erythromycin resistance (Em^r)] with an efficiency of 3×10^5 transformants per μg plasmid DNA. This high efficiency was obtained by the inclusion in the transformation mixture of liposomes composed of cardiolipin and phosphatidyl choline in a molar ratio of 1 to 6 in the presence of 22.5% polyethylene glycol (PEG). This paper also reports an efficient plasmid transfer method between lactic and streptococci and *Bacillus subtilis* by means of protoplast fusion. When *S. lactis* and *B. lactis* protoplasts undergo fusion mediated by exposure to 37.5% polyethylene glycol, plasmid pGKV21 (3.2 MDa; Em^r) was transferred from one host to the other with a frequency of 10^{-3} – 10^{-5} transformants per regenerating recipient protoplast.

2. INTRODUCTION

We have recently described the construction of promoter-screening vectors [1], which are able to replicate in *S. lactis*, *B. subtilis* and *Escherichia coli* [2]. Because a satisfactory transformation protocol for *S. lactis* was not available, *Streptococcus cremoris*-specific promoters could only be selected in *S. lactis* after precloning in *B. subtilis* [1]. Direct gene cloning in lactic acid streptococci requires high transformation efficiencies. It has been reported that in transformation experiments with *Streptomyces* protoplasts [3], yeast protoplasts [4], and transfection of mammalian cells with SV-40 DNA [5], entrapment of the DNA in liposomes increased the delivery of nucleic acids into the host cell as compared to non-aided internationalization of naked DNA. Rodicio and Chater [6] showed that DNA-free liposomes stimulated polyethylene glycol (PEG)-mediated transfection of *Streptomyces* protoplasts and Geis [7] observed that transfecting DNA could be introduced with a higher efficiency into *S. lactis* ssp. *Diacetylactis* by including liposomes in the transformation mixture.

The technique of plasmid transfer by protoplast

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fusions mediated by the fusogenic agent PEG was successfully applied to *S. lactis* [8,9]. More recently, Okamoto et al. [10] showed interspecific protoplast fusions between *S. cremoris* and *S. lactis*.

In this paper we show that liposomes can be used to increase the plasmid DNA transformation efficiency of *S. lactis* protoplasts. In addition, we describe a protocol for the fusion of two phylogenetically widely separated gram-positives, in order to transfer plasmids between the two species.

3. MATERIALS AND METHODS

3.1. Bacterial strains, plasmids and media

The strains and plasmids used are described in Table 1. For preparing plasmid DNA, *B. subtilis* and *E. coli* were grown in TY broth. *S. lactis* strains were cultured in glucose-M17 broth [20] (Difco Laboratories, East Molesey, U.K.). Chloramphenicol was added at final concentrations of 5 µg/ml and 2 µg/ml for *B. subtilis* and *E. coli*,

respectively. Erythromycin was used at a concentration of 5 µg/ml for both *B. subtilis* and *S. lactis*, and 50 µg/ml for *E. coli*.

3.2. Isolation of plasmid DNA

Plasmid DNA was isolated from *E. coli* by the method of Ish-Horowicz and Burke [21]. Plasmid DNA from *B. subtilis* was isolated as described previously [19]. For *S. lactis* the same method was used, except that cells were lysed with a cocktail of 2 mg/ml lysozyme and 100 µg/ml mutanolysin (Sigma Chemical Co.).

3.3. Protoplast formation

Protoplasts of *B. subtilis* were prepared as described by Chang and Cohen [22]. Protoplasts of *B. subtilis*, used as the donors for protoplast fusions, were concentrated 100-fold in S25MM. Protoplasts of *S. lactis* were prepared by the method of Okamoto et al. [23] with minor modifications [1].

3.4. Preparation of liposomes

Small unilamellar vesicles were made by mixing L-α-phosphatidyl choline (Sigma Chemical Co.) and cardiolipin (Sigma Chemical Co.) in various molar ratios. The lipids, dissolved in a mixture of chloroform and methanol were dried under vacuum in a thin-film rotary evaporator, at 45°C. The lipids were suspended in 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA by vortexing for 1 min to give a final lipid concentration of 20 mg lipids/ml. The liposomes were formed by 4 sequential sonications of 15 s each under a nitrogen atmosphere at 0°C. The liposomes were filter-sterilized using a 0.45-µm millipore filter (Schleicher and Schuell, Dassel, F.R.G.).

3.5. Protoplast transformation and fusion

For transformation of *S. lactis* 0.5 ml of protoplasts (derived from approximately 4×10^9 cfu), 1 µg of pGK12 and 5 µl of the liposome suspension were mixed and added subsequently to 1.5 ml of PEG₆₀₀₀ (20–50% solution in S25MM). After incubation for 20 min at room temperature, the transformation mixture was diluted with 5 ml S25MM. For protoplast fusions, 80 µl of *B. subtilis* (pGKV21) donor protoplasts (derived from

Table 1
Bacterial strains and plasmids

Strain or plasmid	Phenotype/genotype	Reference
<i>B. subtilis</i>		
PSL1	<i>leuA8, arg15, thrA, recE4, r_m⁻, m_m⁻</i>	[11]
CU403	<i>Minicell, thy, met</i>	[12]
168	<i>trpC2</i>	[13]
1012	<i>leuA8, metB5</i>	[14]
<i>E. coli</i>		
BHB2600	<i>803, supE⁺, supF⁺, met⁻, r_k⁻, m_k⁻</i>	[15]
<i>S. lactis</i>		
IL1403	Plasmid-free strain <i>lac⁻, prt⁻</i>	[16]
IL1403 (RIF)	Rif ^r IL1403	this work
MG1363	Plasmid-free strain <i>lac⁻, prt⁻</i>	[17]
BU2-60	Plasmid-free strain	
ssp. <i>diacetylactis</i>	Str ^r , Rif ^r , <i>lac⁻, bac⁻</i>	[18]
<i>Plasmids</i>		
pGK12	Em ^r , Cm ^r	[19]
pGKV21	Em ^r , Cm ^r	[1]

approximately 10^9 cfu) in S25MM were mixed with 500 μ l of recipient *S. lactis* protoplasts and immediately added to 1.5 ml PEG₆₀₀₀ solution in S25MM. The fusion mixture was incubated for 20 min at room temperature and diluted by the addition of 5 ml S25MM. After transformation, or fusion, the protoplasts were harvested by centrifugation at $3000 \times g$ for 15 min and resuspended in 1 ml glucose-SM17 and subsequently incubated for 2 h to allow expression of the antibiotic resistance marker. The protoplasts were diluted in glucose-SM17, plated on selective glucose-SM17 agar plates and incubated for one week at 30 °C. If *B. subtilis* was used as the recipient in the protoplast fusion experiments, 250 μ l of *S. lactis* (pGKV21) donor protoplasts (derived from approximately 2×10^9 cfu) were mixed with 250 μ l of *B. subtilis* protoplasts (derived from approximately 0.5×10^9 cfu) in SMMP [22] and immediately added to 1.5 ml 50% PEG solution in S25MM. After 2 min of exposure to PEG at room temperature the mixture was diluted with 5 ml S25MM. Protoplasts were harvested by centrifugation at $3000 \times g$ for 15 min and resuspended in 0.5 ml SMMP, incubated for 2 h to allow expression of the erythromycin resistance (Em^r) marker. From this stage onward appropriate dilutions were plated on DM3 agar [22] containing 5 μ g/ml erythromycin.

4. RESULTS

4.1. Effect of lipid composition of liposomes on transformation of *S. lactis*

In order to determine the parameters for optimal plasmid transformation of *S. lactis*, a small plasmid pGK12 (2.9 MDa) was chosen. This small cloning vector, coding for erythromycin (Em^r) and chloramphenicol resistance (Cm^r), replicates in *S. lactis*, *B. subtilis* and *E. coli* [19].

The lipid concentration of 50 μ g lipids/ml proved to be optimal for the transformation experiments (results not shown). Preliminary results indicated that only neutral- and negatively charged liposomes stimulated protoplast transformation. Based on these data we searched for the optimal negative charge of the liposomes by varying their

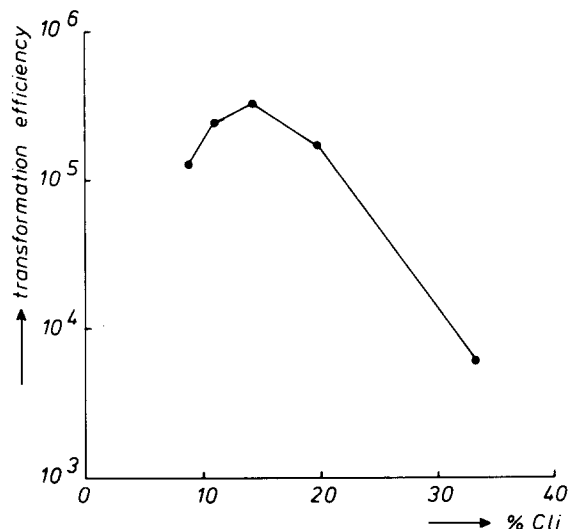


Fig. 1. Effect of lipid composition of liposomes on transformation efficiency (number of transformants/ μ g PGK12). 500 μ l of *S. lactis* IL1403 protoplasts were transformed with 1 μ g pGK12 in the presence of 22.5% PEG in S25MM and liposomes at an end concentration of 50 μ g/ml at room temperature for 20 min in a volume of 2 ml. The molar Cli/Pc ratios are presented as percentage Cli of the total amount of lipids (Cli + Pc) on the horizontal axis. After 2 h expression time at 30 °C in glucose-SM17 medium, Em^r transformants were selected on glucose-SM17 agar containing 5 μ g/ml erythromycin.

lipid composition. Figure 1 shows the effect of the lipid composition of the liposomes on the transformation efficiency of *S. lactis* IL1403 protoplasts. Apparently a sharp optimum exists in the ratio of Cli and Pc with respect to the efficiency of transformation. With less than 10% Cli and in excess of 20% Cli the transformation efficiency dropped sharply. In the absence of liposomes only 200 transformants/ μ g pGK12 were obtained, which is approximately 1600 times less efficient than transformation in the presence of liposomes, consisting of Cli and Pc in a molar ratio of 1 to 6 (14.3% Cli). Under these conditions the transformation efficiency was 3.2×10^5 transformants/ μ g pGK12. The regeneration frequency of the protoplasts in this transformation experiment was 16×10^5 , indicating that 20% of the protoplasts were transformed. The liposomes enhanced transformation efficiency of *S. lactis* MG1364 protoplasts to the same extent (results not shown). Since a molar

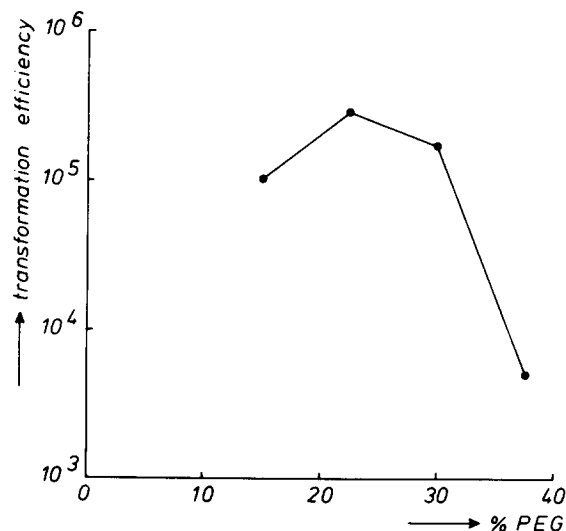


Fig. 2. Effect of PEG₆₀₀₀ concentration on transformation efficiency. Protoplasts of *S. lactis* IL1403 were transformed in the presence of different concentrations of PEG₆₀₀₀ and liposomes composed of Cli and Pc in a molar ratio of 1 to 6 (final lipid concentration: 50 µg/ml). The transformation procedure was the same as described in Fig. 1.

Cli/Pc ratio of 1 to 6 of the liposomes was optimal for the transformation, all subsequent transformation experiments were carried out with liposomes of this composition.

Figure 2 shows the effect of various PEG₆₀₀₀ concentrations on the efficiency of protoplast transformation of *S. lactis*. The figure shows that the optimal concentration was 22.5% PEG. This concentration was the same as found to be optimal in the absence of liposomes [24].

4.2. Plasmid transfer by intergeneric protoplast fusion

To determine whether protoplast fusion can serve as an alternative method for plasmid transfer, avoiding time-consuming DNA isolations, we fused donor protoplasts containing plasmid pGKV21 (3.1 MDa) [1], a derivative of pGK12 with similar properties, to recipient protoplasts by intergeneric and intraspecific protoplast fusions. In addition, we determined the optimal concentration of PEG on the DNA transfer by fusion.

For the protoplast fusion experiments plasmid pGKV21 was both introduced in a minicell forming strain *B. subtilis* Cu 403 [12] by protoplast

transformation according to Chang and Cohen [22] and in *S. lactis* MG1363 by protoplast transformation according to the method described in this paper. The recipient protoplasts of *S. lactis*, *S. lactis* ssp. *diacetylactis* and *B. subtilis* were prepared as described in Section 4. *B. subtilis* whole donor cells, which had escaped from the action of lysozyme, were able to grow on glucose-SM17 plates, forming large mucous colonies. To avoid this, use was made of recipient *S. lactis* strains resistant to rifampicin. The fusion mixtures were plated on glucose-SM17 plates containing 50 µg/ml rifampicin and 5 µg/ml erythromycin to select for the recipient cells which had received plasmid pGKV21 and to prevent growth of the donor *B. subtilis* cells. If *B. subtilis* was used as the recipient in the fusion experiments, the fusion mixture was plated on DM3 plates [22] containing 5 µg/ml erythromycin. On these plates *S. lactis* cells were unable to regenerate and grow. All fusions experiments were performed in the presence of 10 µg/ml pancreatic DNase 1 to exclude PEG-mediated transformation by free plasmid DNA. Figure 3 shows the effect of the PEG concentration on the transfer efficiency of plasmid pGKV21 into *S. lactis* MG1363 after fusion with

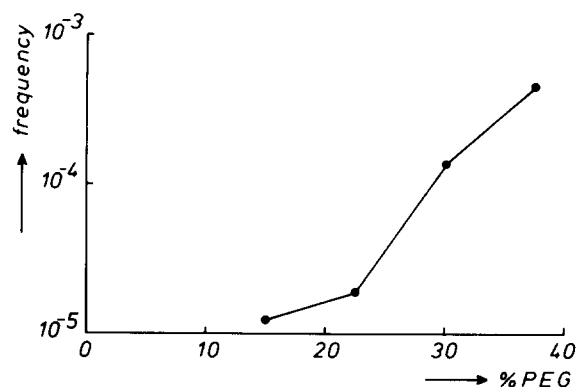


Fig. 3. Effect of PEG₆₀₀₀ concentration on plasmid transfer by protoplast fusion. 80 µl of *B. subtilis* PSL-1 (pGKV21) protoplasts were fused with 420 µl of *S. lactis* MG1363 (Rif^r) protoplasts in the presence of different concentrations of PEG at room temperature for 20 min in an end volume of 2 ml S25MM. After 2 h of expression time at 30 °C in glucose-SM17 medium, fusants were selected on glucose-SM17 agar containing 50 µg/ml rifampicin and 5 µg/ml erythromycin.

donor *B. subtilis* PSL-1 protoplasts. PEG₆₀₀₀ at the highest final concentration used (37.5%), resulted in a high plasmid transfer efficiency. Although at this PEG concentration the optimum was still not reached, further increase of the PEG concentration was impossible, due to the insolubility of PEG. For this reasons, a final concentration of 37.5% PEG was used in all subsequent fusion experiments.

Minicell-forming *B. subtilis* strains distribute plasmids to the minicells. To examine whether this property could be used in an advantageous way to further increase the frequency of plasmid transfer to *S. lactis*, the transfer of Em^r to *S. lactis* was compared after protoplast fusion with a minicell- and a non-minicell-forming *B. subtilis* strain. Table 2 shows that the use of minicell-forming *B. subtilis* CU403 (pGKV21) strain improved the transfer of pGKV21 into *S. lactis* IL1403 (Rif^r) by a factor of 2.3 as compared to the non-minicell-forming *B. subtilis* PSL-1 (pGKV21) strain. Table 2 also shows that plasmid pGKV21 could be introduced in *S. lactis* ssp. *diacetylactis* BU2-60 via protoplast fusion with *B. subtilis* PSL-1 (pGKV21). The frequency of transfer was the same as observed after protoplast fusion with *S. lactis* MG1363 (pGKV21), indicating that pGKV21 plasmids could be introduced in *S. lactis* via intraspecific as well as intergeneric protoplast fusion with the same efficiency. Furthermore, Table 2 shows that plasmids could be transferred in

a very efficient way to *B. subtilis* 168 and *B. subtilis* 1012 in intergeneric fusion with *S. lactis* MG1363 (pGKV21) as donor.

The transferred plasmid DNA by protoplast transformation or fusion was extracted from the Em^r transformants and examined by means of restriction enzyme analysis. No alterations in size and restriction enzyme patterns were observed in agarose-gel electrophoresis, indicating that no changes had taken place in the plasmids (results not shown).

5. DISCUSSION

Plasmid transformation of protoplasts may play an important role in future strain improvement strategies. Several investigators have described efficient protoplast transformation procedures for lactic acid streptococci [24–26]. However, many of these protocols gave variable results in our hands, possibly because of varying degree of trace contamination in the chemicals used. Several reports have dealt with the usefulness of liposomes in the delivery of transfecting and transforming DNA into recipient cells (3–7). In this study, the factor affecting the liposome-assisted transformation of *S. lactis* were examined. Introduction of pGK12 in *S. lactis* IL1403 was most efficient in the presence of 22.5% PEG and liposomes consisting of Cli and Pc in a molar ratio of 1 to 6, at a final lipid concentration of 50 µg/ml. The transformation efficiency under these conditions was 3×10^5 *S. lactis* transformants/µg pGK12. In a comparable transfection system Geis [7] reported an efficiency of $3\text{--}4 \times 10^6$ transfectants/µg phage P008-DNA. The high efficiency of the transfection system may be explained by the fact that no protoplast regeneration is required in transfection. The high transformation frequency obtained in the liposome-assisted transformation system allowed direct gene cloning in *S. lactis* [2].

The optimal PEG concentration for transformation of *S. lactis* in our system was the same as that reported by Kondo and McKay [24] for their system. In the liposome-assisted transfection system for *Streptomyces* protoplasts [6] required a high (50%) PEG₁₀₀₀ concentration similar to that

Table 2

Plasmid transfer by intergeneric and intraspecific protoplast fusions of *S. lactis* strains with *B. subtilis* and vice versa

The efficiency of plasmid DNA transfer is expressed as the number of fusants per recipient.

Recipient strain	Donor strain		
	<i>B. subtilis</i> CU403 (pGKV21)	<i>B. subtilis</i> PSL1 (pGKV21)	<i>S. lactis</i> MG1363 (pGKV21)
<i>S. lactis</i> IL1403	2.8×10^{-5}	1.2×10^{-5}	ND
<i>S. lactis</i> BU2-60 ssp. <i>diacetyl-</i> <i>actis</i>	ND	1.1×10^{-5}	1.0×10^{-5}
<i>B. subtilis</i> 168	ND	ND	7.0×10^{-4}
<i>B. subtilis</i> 1012	ND	ND	1.5×10^{-3}

ND = not determined

for protoplast fusion [27]. The liposomes used in their transfection system were positively charged, which was assumed to favour binding of DNA. Apparently as the result of the present paper shows, negatively charged liposomes are at least as effective in assisting transformation by plasmid DNA. Although the mechanism of enhancement of transformation by the addition of liposomes is unknown, it is conceivable that DNA molecules became associated with the liposomes by means of Mg^{2+} present in the transformation mixture, and that the complexes subsequently fused with the protoplasts after the addition of PEG.

The protoplast fusion experiments show that plasmids can be shuttled between *B. subtilis* and *S. lactis* in an efficient way. This abolishes the need to isolate and purify plasmid DNA. The transfer of plasmid DNA by protoplast fusion described in this paper required high concentrations of PEG (37.5%) as was reported for other protoplast fusion systems [9,10]. A minicell-forming *B. subtilis* strain was superior over a strain forming no minicells as donor of plasmid DNA. However, the increase was moderate, which may be due to the fact that pGKV21, like PGK12, has a low copy number in *B. subtilis* [19], and therefore, may be distributed to only a small fraction of the minicells produced. If this idea is correct, the use of a high copy number shuttle plasmid in the mini-cell producing *B. subtilis* strain might result in a significantly higher frequency of plasmid delivery to lactic streptococci by means of protoplast fusions.

Transfer of pGKV21 from *B. subtilis* PSL-1 to *S. lactis* ssp. *diacetylactis* BU2-60, an intergeneric protoplast fusion, resulted in the same efficiency of plasmid transfer as intraspecific protoplast fusions between *S. lactis* MG1363 (pGKV21) and *S. lactis* ssp. *diacetylactis*. This suggests that a fusant, irrespective of the type of fusion (intergeneric or intraspecific), has the same capacity to regenerate to a complete cell.

The results of the fusion experiments also show that pGKV21 was efficiently transferred from *S. lactis* MG1363 to *B. subtilis* 168 and *B. subtilis* 1012 via protoplast fusion. Recently it was established that plasmids can also be transferred from the phylogenetically unrelated *S. lactis* to

Lactobacillus reuteri by means of protoplast fusion [28]. Provided the plasmid is capable of replication in the two fusion partners, it would seem, therefore, that intergeneric plasmid transfer by means of protoplast fusion between gram-positives may be generally applicable.

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